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Importance of the Methyl Group at C(10) of Squalene for Hopene Biosynthesis and Novel Carbocyclic Skeletons with 6/5 + 5/5 + (6) Ring System(s)

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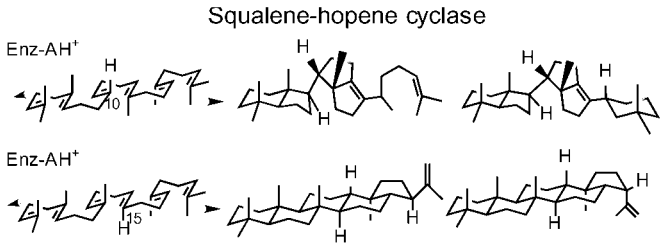
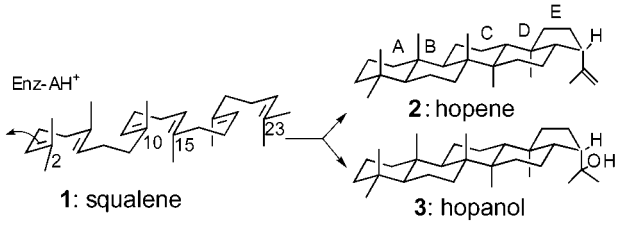
ABSTRACT



Incubation of (6*E*,10*E*,14*E*,18*E*)-2,6,10,19,23-pentamethyl-tetracosa-2,6,10,14,18,22-hexaene with *Alicyclobacillus acidocaldarius* hopene cyclase afforded four products having two types of carbocyclic skeletons, i.e., two hopane products and two products having an unprecedented carbocyclic skeleton of 6/5 + 5/5 +6 pentacyclic and 6/5 + 5/5 tetracyclic ring systems. The former two hopane skeletons were formed from the bioconversion of C(15)-desmethylsqualene and the latter two skeletons from that of C(10)-desmethylsqualene.

In the past several years, the catalytic mechanism of squalene−hopene cyclase (SHC) from *Alicyclobacillus acidocaldarius* has been rapidly unraveled.[1] This prokaryotic cyclase converts a flexible squalene molecule **1** into pentacyclic hopene **2** and hopanol **3** (**2**:**3** = ca. 5:1) having the 6/6/6/6/5-fused ring system, in which all of the six-membered rings have a chair structure (Scheme 1).[1,2] The cyclization mechanism is analogous to that of eukaryotic oxidosqualene cyclases.[2] We have established that the polycyclization consists of eight reaction steps[1] and that the ring expansion reaction occurs twice for the formation of the six-membered C- and D-rings of **2** on the basis of the successful trappings of five-membered C- and D-ring intermediates (Markovnikov adducts) by using site-directed mutants and substrate analogues.[3] Previously, we have shown by using C(23)-desmethylsqualene that the isopropylidene moiety is indispensable for the correct folding of **1** both to form the five-membered E-ring and to initiate the polycyclization reaction;[4] incubation of the norsqualene with the native SHC gave products having a six-membered E-ring (i.e., a tetrahymanol

Scheme 1. Polycyclization Pathway of Squalene **1** into Hopene **2** and Hopanol **3** by Squalene−Hopene Cyclase



(1) (a) Review for squalene−hopene cyclase. Hoshino, T.; Sato, T. *J. Chem. Soc., Chem. Commun.* **2002**, 291. (b) Schmitz, S.; Füll, C.; Glaser, T.; Albert, K.; Poralla, K. *Tetrahedron Lett.* **2001**, *42*, 883.

(2) Reviews for oxidosqualene and squalene cyclases. (a) Wendt, K. U.; Schulz, G. E.; Corey, E. J.; Liu, D. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 2812. (b) Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189.

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skeleton with a 6/6/6/6/6-fused A/B/C/D/E-ring system), but afforded no enzymic product that is assumed to be formed by starting the cyclization reaction from the methyl-deficient side. However, the role of the methyl groups at positions[5] of 6, 10, 15, and 19 for the cyclization cascade has remained unknown. We planned to address the question of how the methyl groups at the central part of **1** affect the polycyclization cascade. Here, we describe that when the Me at C(10)[5] is missing, the polycyclization cascade is dramatically altered to afford unprecedented cyclization products consisting of the 6/5-fused (A/B) and the 5/5-fused (C/D) ring system, in which the B- and C-rings are not fused. This finding strongly indicates that the C(10)-Me[5] is essential to the correct folding of **1** for completion of the normal polycyclization reaction leading to **2** and **3**.

Norsqualene **4** lacking the methyl group at the central part of **1**, (6*E*,10*E*,14*E*,18*E*)-2,6,10,19,23-pentamethyl-tetracosa-2,6,10,14,18,22-hexaene, was prepared according to the synthetic scheme described in Supporting Information.

With the cell-free homogenates from 4 L of culture of the recombinant *E. coli* encoding the SHC,[6] 50 mg of **4**, emulsified with Triton X-100, was incubated at catalytically optimum conditions (pH 6.0, 60 °C) for 16 h. The enzymic products were extracted with hexane. The GC and TLC showed four enzymic products. Triton X-100 was removed by passing the products through a short SiO_2 column (hexane). The repeated SiO_2 column chromatography (5% $AgNO_3$, 100:0.01 hexane/EtOAc) gave **5** and **6** as an oil, while **7** and **8** were obtained as a solid in a pure state, the elution order being **8**, **7**, **6**, and **5**. The product amounts of **5**−**8** and the recovered **4** were estimated to be 16.8, 3.8, 10.5, 7.3, and 9.0 mg, respectively, from the GC analysis.

The detailed NMR analyses (COSY 45, HOHAHA, NOESY, DEPT, HMQC, and HMBC) revealed the structures of all the enzymic products (Figure 1). Products **5** and **6** have a novel skeleton having a 6/5 + 5/5 ring system, which was unequivocally determined by HMBC and NOESY data as shown in Figure 2. This ring system was further supported by the fragment ion *m*/*z* 285 (∼100%) in the EIMS spectra of **5** and **6**. The strong NOEs of H-10 with Me-25 and Me-26 verified the β-orientation for both H-10 and Me-26. A clear NOE of H-5 with H-8 indicated that both protons were in the α-orientation, but no NOE was observed between H-8 and H-10. H-18 of the E-ring in **6** must be arranged in an axial disposition, because clear NOEs were observed for H-18 with H-20 and Me-28. The stereochemistry at C(18) of **5** and **6** has yet to be established. Products **7** and **8** had a fused pentacyclic structure, which was established by the

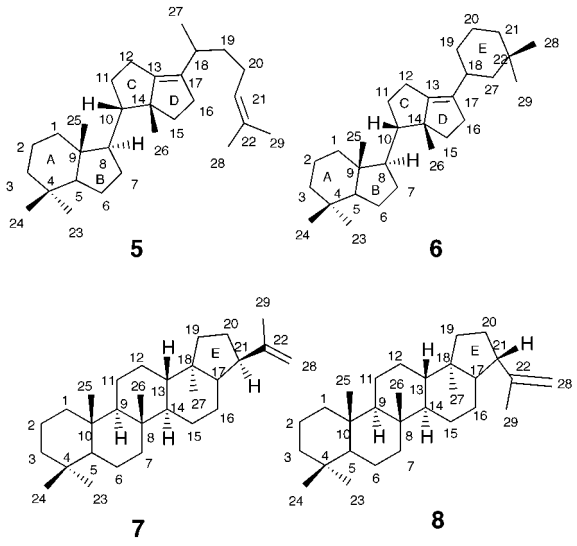


Figure 1. Structures of enzymic products **5**−**8**.

NMR analyses. Product **7** has an isohopane skeleton,[7] lacking a methyl group at C(14).The eminent ion *m*/*z* 191 in the EIMS spectra of **7** and **8** proved the 6/6-fused A/B-ring (Scheme 2).[8] However, the stereochemistry at C(21) of **7** was opposite to that of natural type **8**; a strong NOE of H-21 with Me-27 was observed for **7**, whereas no NOE was observed between them for **8**.

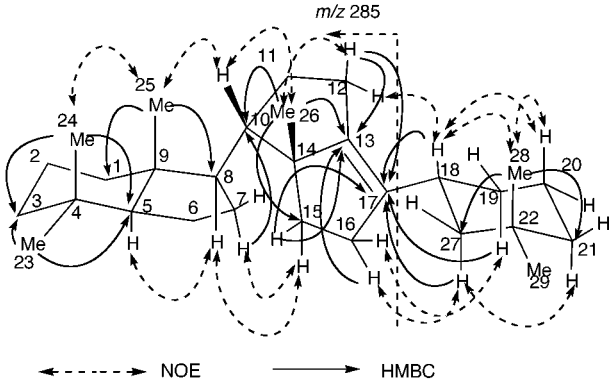


Figure 2. Selected HMBC and NOE correlations of **6**.

Previously, we have shown that an isopropylidene moiety of **1** is required to start the polycyclization reaction. No cyclization occurs when one of the two methyl groups is missing.[4] Analogue **4** has two isopropylidene moieties on the left and right terminal sides of the squalene backbone. Thus, **4** was cyclized not only as 10-desmethylsqualene **4a**[5]

(3) (a) Sato, T.; Abe, T.; Hoshino, T. *J. Chem. Soc., Chem. Commun.* **1998**, 2617. (b) Hoshino, T.; Kouda, M.; Abe, T.; Ohashi, S. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 2038.

(4) Hoshino, T.; Kondo, T. *J. Chem. Soc., Chem. Commun.* **1999**, 731.

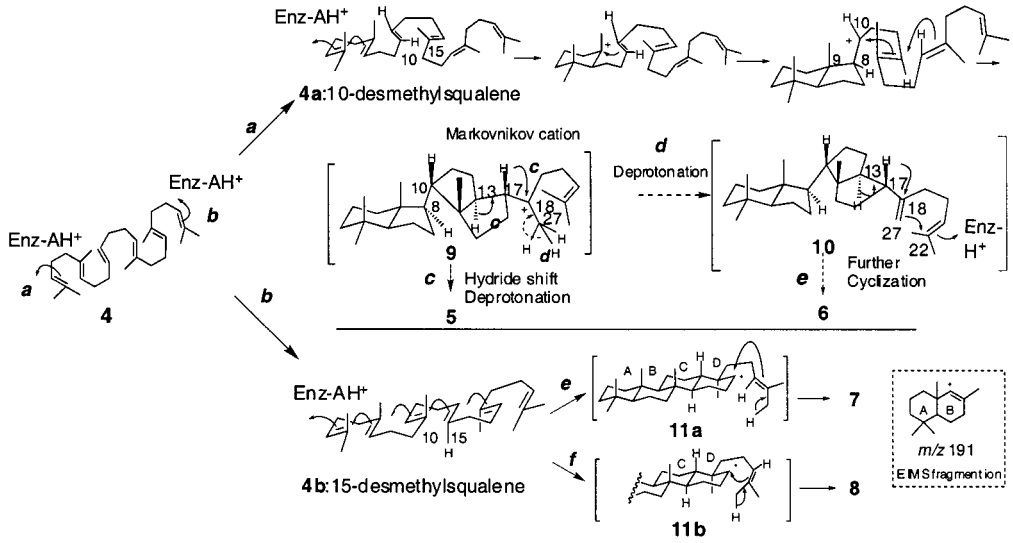
(5) The numbering of norsqualenes discussed in this paper is enumerated from the direction of the cyclization of norsqualenes strats. Because analogue **4** was cyclized from the both terminal isopropylidene moieties (Scheme 2), this numbering system allows the discrimination of C(10)-**4a** from C(15)-desmethylsqualenes **4b**. However, both **4a** and **4b** should be named 15-desmethylsqualene according to the nomenclature rule.

(6) Sato, T.; Kanai, Y.; Hoshino, T. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 404.

(7) Ageta, H.; Shiojima, K.; Suzuki, H.; Nakamura, S. *Chem. Pharm. Bull.* **1993**, *41*, 1939.

(8) Shiojima, K.; Arai, Y.; Masuda, K.; Takase, Y.; Ageta, T.; Ageta, H. *Chem. Pharm. Bull.* **1992**, *40*, 1683.

Scheme 2. Polycyclization Pathway of **4** into Products **5**−**8**



to give **5** and **6** via path *a* but also as 15-desmethylsqualene **4b**[5] to afford **7** and **8** via path *b*, as shown in Scheme 2. The product ratio of **5**, **6**, **7**, and **8** was 4.4:1:2.8:1.9, indicating that both paths *a* and *b* had proceeded in a nearly equal ratio. The Markovnikov cation **9** produced in path *a* could undergo the 1,2-shift of hydride of H-17 to the C(18)-cation, followed by deprotonation from H-13, resulting in the introduction of the double bond between C(13) and C(17) (path *c*). H-13 and H-17 must be arranged in antiperiplanar geometry to allow the double-bond formation. The β-orientation of Me-26 indicates the α-arrangement of H-13, because all the double bonds of **4** have an (*E*)-geometry; thus, H-17 of **9** must be in the β-orientation. The proton elimination from Me-27 of **9** (path *d*) could give **10** having a methylidene moiety, which could undergo the sequential reactions of further cyclization, hydride shift, and deprotonation to give **6**. Intermediate **10** was not trapped, indicating that this further cyclization must be fast, which may have been catalyzed by the acidic amino acid(s) located near the substrate entrance.[9] The reaction mechanism shown in paths *c* and *e* may suggest 18*S* for **5** and 18*R* stereochemistry for **6**.[10] Product **8** was produced via **11b** according to the normal folding conformation, whereas **7** was produced via the incorrectly folded structure **11a**. The dramatically altered cyclization pathway of **4a** (path *a*), compared to that of **4b** (path *b*), indicated that C(10)-Me plays a more crucial role for construction of the hopane skeleton than C(15)-Me.

Why were unprecedented carbocyclic **5** and **6** produced from **4a**? It is difficult to get a clear answer to this question at the present time, but one possible explanation may be as follows. The accurate cavity size or binding site to accept C(10)-Me of **1** may be involved in the SHC; a strong interdigitation between the C(10)-Me and its binding site may enable **1** to adopt a six-membered chair structure for the B-ring formation and to direct β-orientation for the C(10)-Me during the polycyclization process (Scheme 1). However, the less bulky hydrogen at C(10) of **4a** could not precisely interact with the binding site; in turn, the methyl group at C(15) of **4a** could be strongly captured by the binding site intrinsic to C(10)-Me of **1** through the folding conformation shown in path *a*. Thus, the C(15)-Me could have a β-orientation, in contrast to the α-orientation for the bioconversion of **1**→**2** (Scheme 1). On the other hand, **4b** having C(10)-Me could adopt the normal folding structure through the correct fitting of the C(10)-Me with the binding site inherent to the C(10)-Me of **1** (path *f*), resulting in the production of hopene homologue **8**. However, substitution with a less bulky hydrogen at C(15) could influence to some extent the normal folding around the E-ring formation site to give **7** (path *e*).

(9) There may exist the possibility that DXDDTA motif, which is responsible for the initiation of the polycyclization (Sato, T.; Hoshino, T. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 2189), could also participate in the cyclization of intermediate **10**. However, this possibility is unlikely, because **10** must again have access to the acidic motif to produce **6** after exiting the cyclase cavity. In this case, **10** should be detected in the reaction mixture.

(10) To gain the opposite stereochemistry (18*R* for **5** and 18*S* for **6**), a large rotation of the side chain through the C(17)−C(18) axis of **9** and **10** must occur prior to the hydride shift of C(17) to C(18). This large motion is unlikely inside the enzyme cavity. Through the biosynthetic studies of lanosterol, Corey et al. have proposed that the side chain at C(17) of protosteryl cation must have a β-orientation to get the 20*R* stereochemistry of the lanosterol skeleton, in contrast to Cornforth's hypothesis for the 17α-orientation (Cornforth, J. W. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 903).[2b] Corey's idea is based upon the successful trappings of the products having the 17β-oriented side chain by using substrate analogues, and they explained the reason for the 17β-orientation: 20*R* stereochemistry can be readily obtained by the least motion (<60°) for the 17β-oriented side inside the enzyme cavity, whereas the 17α-oriented one must have the large rotation of 120° (Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1991**, *113*, 4025).[2b] Thus, we assign 18*S* and 18*R* for **5** and **6**, respectively, due to the least motion of the side chain. To gain the opposite stereochemistry at C(10) and C(14) in **5** and **6**, a large rotation (180°) is also required around the C(8)−C(10) axis during the polycyclization; thus, this possibility also is unlikely. Furthermore, the lifetime of the secondary C(10)-cation formed during the polycyclization is short, in contrast to stable tertiary C(18)-cation in **9**, because no abortive cyclization product having a bicyclic 6/5-fused ring system, which is generated from C(10)-cation, was trapped. Thus, the formation of the C-ring must be fast, and the epimerization at C(10) would not occur. The stereochemistry at C(18) and C(10) in **5** and **6** must be further ascertained by X-ray analysis to validate our assumption.

Corey et al. have shown that the C(10)-Me of (3*S*)-2,3-oxidosqualene is important for the normal polycyclization pathway in lanosterol biosynthesis. Lack of the two methyl groups at both C(10) and C(15) led to the abnormal cyclization product having a tricyclic 6/6/5-fused A/B/C-ring system, in which the B-ring had a chair structure, despite lanosterol synthase adopting a twist-boat form for the B-ring formation.[11] The absence of the C(15)-Me had no influence on the polycyclization pathway leading to the formation of a lanosterol homolog.[12] It was suggested that the hydrophobic group responsible for the interaction with the C(10)-Me is involved in lanosterol cyclase.[11] Introduction of a slightly bulkier ethyl group at C(10) into 2,3-oxidosqualene also significantly influenced the biosynthetic pathway of lanosterol;[13] 10-ethylated-2,3-oxidosqualene was converted into the three abortive cyclization products consisting of two monocycles and one 6/6/5-fused tricycle in addition to the fully cyclized lanosterol homologue.[13c] Therefore, the bulk size at C(10) must be accurate to correctly interact with the hydrophobic group of the cyclase; a hydrogen atom is small, but an ethyl group is large.

The mutation experiments of squalene and oxidosqualene cyclases also have highlighted the appropriate steric bulk size at the active sites; modification of the bulk sizes for the SHC (e.g., mutations of I261→A and L607→W) led to the creation of unnatural natural products[1,14] and that for the cycloartenol synthase allowed lanosterol production.[15] The flexible and linear molecules of **1** and oxidosqualene can adopt numerous folding structures, but the steric bulk sizes of active site residues involved in triterpene cyclases may well direct a specific folding conformation to the substrates for producing a given triterpene skeleton.

In conclusion, we have shown that the methyl group at C(10) is essential to the correct folding of **1** for hopene biosynthesis. To the best of our knowledge, the triterpene skeletons of **5** and **6** possessing 6/5 + 5/5 + (6) ring systems have not hitherto been reported. The norsqualene lacking a methyl group at C(6) or C(19) gave a hopane skeleton via the normal polycyclization pathway (unpublished results).[1a] Thus, the following three methyl sites of **1** are crucial for the correct folding in the hopene biosynthesis: (1) the site for initiation,[4] (2) the central site at C(10), and (3) the termination site.[4] To validate the hypothesis for involvement of the binding sites in the SHC, mutagenesis experiments are necessary in which the steric bulk sizes of the active site residues (hydrophobic groups) are modified.

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Supporting Information Available: Synthetic scheme of **4**, incubation conditions, EIMS, NMR data, and specific rotation of **5**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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